agriculture

MDPI

Article

The Influence of Sewage Sludge and Fly Ash Fertilization on the Total Number of Bacteria (TNB) and *Bradyrhizobium* Species in Soybean Agroecosystem

Izabella Pisarek * and Katarzyna Grata

Institute of Environmental Engineering and Biotechnology, Faculty of Natural Sciences and Technology, University of Opole, 11 Pl. Kopernika St., 45-040 Opole, Poland
* Correspondence: izapis@uni.opole.pl

Abstract: The aim of this study was to determine the impact of different chemical properties in soil, including changes in magnetic susceptibility and the impact of humic substances from sewage sludge and fly ash on the microbial community in the soybean agroecosystem. A field experiment was carried out using the random plot method on Cambisol with a texture of silt loam. The experiment consisted of 24 plots (six treatments and four replications) using different dozes of sewage sludge and fly ash. The following physicochemical and chemical analyses were performed in the soil samples: pH in KCl, conductivity (λ), total content of heavy metal, magnetic measurements and fractional composition of organic matter. The TNB and the number of the species of *Bradyrhizobium* in the field cultivation of the soybean variety Lissabon were evaluated using the spread plate method. The total content of heavy metals (Cu, Zn, Cd, Ni, Pb, Cr, Hg, Fe) in the analyzed treatments showed a significant difference between them. The study of the magnetic parameter χ indicated a significant differentiation between treatments from 34.0 to 65.8 $\times$ $10^{-8}\cdot m^3\cdot kg^{-1}$. High correlation coefficients between χ and Fe (r = 0.789), Zn (r = 0.653), Cr (r = 0.617) and TOC (r = 0.949) indicated that the source of these elements was external organic matter. Biological tests (TNB and species of *Bradyrhizobium*) in different experimental treatments indicated significant relationships between them and showed the resistance of the microbial community in the field cultivation of Lissabon soybean to heavy metal contamination from sewage sludge and fly ash. The study confirmed that external organic matter, such as sewage sludge, can be used as an alternative to natural fertilizers for soybean production.

Keywords: soybean agroecosystem; *Bradyrhizobium* species; magnetic susceptibility; humic substances

Citation: Pisarek, I.; Grata, K. The Influence of Sewage Sludge and Fly Ash Fertilization on the Total Number of Bacteria (TNB) and *Bradyrhizobium* Species in Soybean Agroecosystem. *Agriculture* **2023**, *13*, 201. https://doi.org/10.3390/agriculture13010201

Academic Editors: Jolanta Kwiatkowska-Malina, Grzegorz Malina and Ram Prasad

Received: 10 October 2022
Revised: 2 January 2023
Accepted: 10 January 2023
Published: 13 January 2023

1. Introduction

The cultivation of leguminous plants benefits agricultural production both in ecological and economic terms [1]. The first of these is related to the improvement of the habitat quality; the second is to the profitability of production [2]. Soybean (*Glycine max* L.) is one of the most important leguminous plants cultivated in the world. The main advantage of its cultivation is the binding of biological bacterial nitrogen (N_2) in cereal and fodder grains, which increases the yield potential by reducing the use of exogenous fertilizers. Due to climatic and geographical conditions, only cultivars of soybeans grown or tested for their usefulness in Poland may be grown there. Since soybean is a short-day plant with high thermal requirements, most excellent yielding varieties grown in South America do not mature in Poland. For cultivation under Polish weather conditions, the best varieties are those which during the growing season require the sum of daily temperatures up to 2200 °C, and whereby their growing season does not exceed 140 days [3,4]. Furthermore, the soybean yield needs to include chemical and biological methods in the agricultural technology cycle. In such a system of cultivation, the use of fertilizers should be subordinated to the biological activity of the habitat, mainly due to the economics of production and the quality of the environment [1,5,6]. The application of organic matter in the soil stimulates biological activity and creates biotic relations in the environment [7–9]. In addition, external organic matter plays a special role in the balance of humus in the soil. External organic matter is defined as all organic material which is introduced into the soil, including sewage sludge, to improve soil quality and to preserve future production potential [8]. Soils are characterized as having a high integration capacity. Soils are able to assimilate and transform external organic matter, leading to a synthesis of humic substances and organic–mineral complexes [8]. Many studies emphasize the positive impact of sewage sludge on the diversification of microflora and microfauna. Its high content of easily assimilable forms of nitrogen, phosphorus and micronutrients is one of the parameters determining its agricultural usability and qualifies it as a valuable conditioner [10].

The scientific research also shows [11] that the addition of fly ash to sewage sludge visibly improves its physical, chemical and biological properties and simultaneously simplifies its agricultural use. The main components of fly ash are oxides of silicon, aluminum and iron. Fly ash contains large amounts of calcium, magnesium and potassium as well as microelements that determine its fertilizing value. Their presence causes anomalies of the magnetic susceptibility of soils, which is correlated not only with anthropogenic factors, but also with the properties of the parent rock and physicochemical processes occurring in the soil, as well as the activity of bacteria [12].

Magnetometry is a method used to determine slight changes in soils, and similarities or differences between soil types [13,14]. The amount and distribution of oxides, hydroxides and elements from the group of ferromagnetics in the soil are indicators of both the intensity of the soil-forming process and anthropopression. Their mobility in soils depends on the quality and quantity of humic acids. A profile of the concentration, distribution and type of ferromagnetic elements in the soil can be made by measuring magnetic properties [13]. Therefore, it is increasingly common in the scientific research to use the magnetic susceptibility of soils as an indicator of the degree of their pollution due to the use of unconventional soil conditioners. Research has shown that traditional soybean cultivation activates a general decrease in the nutrient content of the soil, and afterwards modifies its biotic ratios [7,15–17]. Therefore, in leguminous crops, agrotechnical treatment is necessary to enrich the soil with macro- and microelements. The efficiency of soybean cultivation is related to the inclusion of chemical and biological methods in the agricultural technology cycle [15,16,18–22]. In the cultivation of legume plants, local microorganisms are characterized by a high metabolic activity and as a result have an impact on biochemical processes related to the transformation of carbon compounds. The cells of microorganisms involved in the transformation of soil organic matter contain about 1000 enzymes which depend on the presence of ions and the pH of the environment for their activity [22]. Plants, during their growth, are in close and continuous contact with the microorganisms present in the vicinity of the roots, i.e., the rhizosphere [23–25]. Rhizodeposits (secretions, root exudates, mucus) released by plants constitute easily digestible substrates for microorganisms [26–28]. These microorganisms exert a number of positive effects on the plant through direct and indirect mechanisms and are referred to as plant-growth-promoting rhizobacteria (PGPR). PGPR microorganisms, especially the ones belonging to the genera *Pseudomonas*, *Bacillus*, *Azospirillum* and *Trichoderma*, are used as components of biofertilizers, biopreparations and bio-activators. Moreover, co-inoculation of *Bradyrhyzobium* spp. with plant-growth-promoting bacteria can improve soil fertility, increase crop growth and yield and is environmentally friendly [26–29]. Microorganisms also act as plant growth promoters due to their ability to convert atmospheric nitrogen to ammonia and its further incorporation into biomolecules, phytohormone synthesis, iron chelating by siderophores, phosphorous solubilization and amino acid and indole acetic acid (IAA) production. Among other benefits, it is noted that some PGPR produce secondary antimicrobial metabolites, among others, antimicrobial compounds, volatile organic compounds (VOCs) and cell-wall-degrading enzymes (e.g., cellulases, chitinases, pectinases, proteases). Hydrolytic enzymes are related to the degradation of the cell wall or fungal cell membrane, protecting plants against pathogens. They also cause the degradation of extracellular virulence factors and stimulate the plant's immune system. Moreover, they are involved in biocontrol against pathogens and are of key importance in the processes related to the colonization of plant surfaces and invasion through plant cell walls and the intercellular space. These substances may have a positive effect on the plant, but most of all they allow bacteria to survive in the rhizosphere environment under conditions of high competition. Through a series of positive reactions, the rhizosphere microorganisms promote plant growth by increasing the availability of essential nutrients, inhibiting the growth and spread of phytopathogens and inducing resistance to various biotic and abiotic factors [30–34]. Our previous work showed [7] that changes in physicochemical and chemical properties of habitats visibly modify biotic relationships in soils, which contributes to the diversity of the quantity and quality of nutrients in the soil solution and the sorption complex through their immobilization, leaching of easily soluble forms or erosion. Fertilizer applications of sewage sludge, fly ash or their mixtures not only use their fertilizing properties, but can also modify the biotic relationship system [35,36]. The metabolites of microorganisms can create chelates or permanent deposits with metals and, as a result, deactivate their toxic effects. According to the research [8,19,22,34,37], regardless of the place of xenobiotic deactivation (inside the cell or as a result of absorption on its surface), its presence in the environment influences the number and activity of microorganisms. The climate and richness of soils are not the only factors limiting the area of soybean cultivation in Poland. Above all, there is a different microflora of the native agrohabitat, where symbiotic bacteria of soybeans (*Bradyrhizobium japonicum*) are very rare. Inoculation with symbiotic *Bradyrhizobiaceae*, which can assimilate nitrogen, is a simple and effective method of improving the efficiency of soybean cultivation. It is an example of successful symbiosis affecting the nitrogen binding intensity [5]. In many scientific works [1,2,15,18,21,28,38,39], finding the abundance of *B. japonicum* is considered to be an important method for determining the biological activity in soybean cultivation, under both field and experimental conditions and in various fertilizer variants. Furthermore, TNB in the soil is usually regarded as an early indicator of changes in soil quality and soil fertility [40]. Therefore, the aims of this study were (1) to analyze the changes in chemical parameters of soil and their magnetometry in analyzed treatments with sewage sludge and fly ash, and (2) to investigate the effects of analyzed treatments on the TNB and *Bradyrhizobium* spp. community.

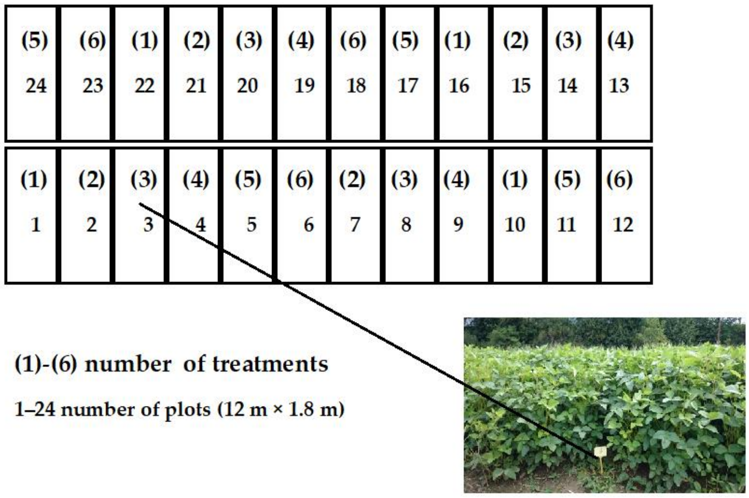
2. Materials and Methods

2.1. Field Experiments

The field experiments were carried out on the arable lands of the Experimental Station for the Evaluation of Varieties in Glubczyce (they were conducted by Marta Bednarczyk), using the random block method on Cambisol with the granulometric composition of silt loam: ∅ in mm: >2 = 0.5%; 2–0.05 = 12%; 0.05–0.002 = 70%; <0.02 = 50%; <0.002 = 18% (according to PN-Z-19012:2020-02). The experiment included 24 plots in six treatments and four repetitions (Scheme 1): (1) control; (2) sewage sludge (80 Mg/ha); (3) sewage sludge (160 Mg/ha); (4) sewage sludge + fly ash (80 Mg/ha + 40 Mg/ha); (5) sewage sludge + fly ash (160 Mg/ha + 80 Mg/ha); (6) fly ash (40 Mg/ha).

2.2. Soil

As far as the soil samples taken at the stage of full soybean maturity are concerned, the following physicochemical and chemical analyses were performed: pH in KCl (PN-ISO 10390:1997); conductivity (λ) of aqueous extracts (5:1) via the conductive method (PN-ISO 11265:1997); analysis of the total heavy metal content in the soils was determined with the AAS method. The sample digestion for the determination of heavy metals was made using a microwave MARS X (CEM Inc., Matthews, NC, USA 2007). The content of heavy metals (copper (Cu), zinc (Zn), cadmium (Cd), nickel (Ni), lead (Pb), chrome (Cr), iron (Fe)) was determined using an atomic absorption spectrometer (THERMO iCE 3000, ThermoFisher Scientific Inc., Waltham, MA, USA, made in China 2009) according to PN-ISO-11047:2001; the content of mercury (Hg) was determined using the AMA 254 mercury analyzer; the content of organic carbon was determined by means of an Analyzer Multi N/C 3100 (Analyticaljena Inc., Jena, Germany 2013) according to PN-ISO 14235:2003; fractional composition of humic substances (HS), humic acids (HAs) and fulvic acids (FAs) were determined as recommended by the American Colloid Company Procedure 3009 [41].



Scheme 1. Scheme of field experiment.

Soil magnetic measurements were conducted using the method described by Zawadzki et al. [14] and Goluchowska et al. [42]: measurement of volume and low field magnetic susceptibility (κ) of samples to be made using a Bartington susceptibility meter equipped with a laboratory sensor MS2B (Bartington Instruments Ltd., Witney, OX, UK 2010), followed by calculation of specific (mass) magnetic susceptibility (χ) using the formula:

$$\chi = \frac{\kappa}{\rho} \quad (1)$$

where:

χ—specific (mass) magnetic susceptibility ($\times 10^{-8} \cdot m^3 \cdot kg^{-1}$);

κ—volume, low-field magnetic susceptibility (dimensionless quantity in SI system of units);

ρ—volume density of a sample in natural stage ($kg \cdot m^{-3}$).

All analyses were conducted in four replicates. The results presented in the tables are their arithmetic mean. Statistical calculations: correlation coefficient (r) and LSD were found using Excel and statistical program (n = 24, level of significance 0.05 and 0.01).

2.3. Sewage Sludge and Fly Ash Properties

Properties of sewage sludge and fly ash were analyzed using the methods described above, and they showed the following parameters:

Sewage sludge: pH 6.8; λ 2250 $\mu S \cdot cm^{-1}$; total content of heavy metals in $mg \cdot kg^{-1}$: Fe 27,060; Cu 95.6; Zn 1054; Pb 95.2; Cd 3.6; Cr 81.0; Ni 21.2; Hg 2.25; χ 564 $\times 10^{-8} \cdot m^3 \cdot kg^{-1}$.

Fly ash: pH 11.8; λ 2970 $\mu S \cdot cm^{-1}$; total content of heavy metals in $mg \cdot kg^{-1}$: Fe 49,390; Cu 145.0; Zn 760.0; Pb 296.0; Cd 3.8; Cr 60.0; Ni 73.0; Hg 1.55; χ 729 $\times 10^{-8} \cdot m^3 \cdot kg^{-1}$.

2.4. Soybean

In the field experiment, the Lissabon soybean variety was used. It is characterized by a very high yielding potential and resistance to lodging and weed infestation in the Polish climate. The seeds were inoculated using HiStick® Soy (BASF Sp.z o.o., Warsaw, Poland), containing live bacteria of the *Bradyrhizobium* which cause a more efficient assimilation of nitrogen from the air in the soil in plants. HiStick® Soy contains at least 2 (2×10^9) billion live cells of *Bradyrhizobium japonicum*. To inoculate 100 kg of soybeans via dry inoculation before sowing, 400 g of HiStick® was used.

2.5. Biological Tests

As far as the soil samples (ectorhizosphera) are concerned, the determination of the TNB (a) and the total number of the *Bradyrhizobium* species (b) was made using the culture plate method as follows:

(a) From the collected soil samples, 5 g were taken and suspended in 45 mL of physiological saline and shaken for 20 min. A series of tenfold dilutions were made and spread onto TSA (tryptic soy agar). The culture was grown at 30 °C for 48 h. The colonies from individual dilutions in the tested samples were counted and the results were given as the mean of replicates ± SD in colony-forming units (cfu) per 1 g of fresh soil mass. The experiment was carried out in six replicates.

(b) The number of colonies formed in the plates was enumerated and the average number of the colony-forming unit (CFU/g and CFU/root nodule) was calculated using the following formula [32]:

$$\text{CFU/gorCFU/rootnodule} = \frac{\text{number of colonies}}{\text{volume of culture plated in mL}} \times \text{dilutionfactor} \quad (2)$$

Four root nodules were separated from the tested samples, which were pre-rinsed several times under running water. Subsequently, the surfaces of the root nodules were sterilized using ACE (1:4 dilution was prepared in sterile distilled water). The root nodules were shaken for 20 min and then rinsed several times with sterile distilled water. The root nodules were dried up using sterile tissue and placed in a sterile mortar. With the use of a sterile scalpel, they were pre-cut into smaller pieces and homogenized in a small amount of physiological fluid. A series of tenfold dilutions from the prepared homogenates were made and then spread on a modified Rhizobium medium (RH) with the following composition (g/L): mannitol 10; K_2HPO_4 0.5; $MgSO_4$ 0.2; NaCl 0.1; yeast extract 1.0; agar 20.0. After sterilization, cycloheximide 200 mg/L and neomycin 2.5 mg/L were added to the medium [43,44]. The cultures were grown at 30 °C for 48–72 h. The result was given in cfu/root nodule as the mean ± SD of four replicates. The results were statistically calculated using the one-way analysis of variance with Tukey's test at the significance level of 0.05.

3. Results and Discussion

A problem with the agricultural use of sewage sludge may be the presence of inorganic and organic toxic components [8,10,45,46]. Of inorganic pollutants, the heavy metal content is particularly noteworthy since its excessive amount in the soil poses a potential risk to the environment [39,47]. The agricultural use of fly ash also raises a lot of controversy because of the potential hazard to the environment, mainly due to the migration of heavy metals [11,42]. However, the alkaline pH limits the mobility of these elements and may be an alternative to liming and sewage sludge hygienization. The total content of heavy metals copper (Cu), zinc (Zn), cadmium (Cd), nickel (Ni), lead (Pb), chrome (Cr), mercury (Hg) and iron (Fe) in the analyzed treatments, as compared to the limit values for uncontaminated soils, indicates their natural content [8,45] despite the application of sewage sludge and fly ash according to the experimental design (see Section 2.3). Simultaneously, their content shows quantitative variation depending on the variant of the experiment with significant relationships between treatments (Table 1). A significant increase in Fe, Zn and Cr in soils of Treatments 4, 5 and 6 was seen in relation to the control (Treatment No 1). Significant differences in Hg content were recorded across all treatments. However, the lowest Hg content was noted on plots enriched with fly ash only (Treatment No 6). There were no significant correlations between the amounts of Cu, Pb and Ni in the treatments.

Table 1. Chemical properties of analyzed soil samples.

No of Treatment	Total Content of Elements on Average [mg·kg^{-1}]								χ [$\times 10^{-8}$·m^3·kg^{-1}] (fd in%)	pH	Conductivity [μS·cm^{-1}] λ
	Fe	Cu	Zn	Pb	Cd	Cr	Ni	Hg			
1	1.9	17.4	56.2	51.0	0.541	25.0	15.7	0.233	34 (2.3)	5.0	96.0
2	2.1	17.9	59.1	51.8	0.473	26.4	14.2	0.349	38 (4.7)	5.2	103.0
3	2.3	20.3	57.9	40.4	0.591	25.3	13.3	0.254	34.1 (4.7)	5.5	103.5
4	2.5	18.9	59.8	37.8	0.574	26.7	15.2	0.260	65.8 (2.5)	5.9	155.1
5	2.4	18.6	60.8	34.1	0.574	25.3	14.5	0.236	49 (4.8)	6.6	259.0
6	2.5	17.9	58.1	36.3	0.693	25.7	15.5	0.230	52.4 (4.8)	6.7	226.0
LSD	0.21	7.68	1.59	9.78	0.06	0.62	0.83	0.04	11.48	0.65	64.12

LSD—low significant difference at $p = 0.05$; $n = 24$.

Magnetometry is a suitable method for soil diagnostics and for finding the degree of contamination. Magnetic properties (ferro and ferrimagnetic properties) of soils depend both on the original lithogenic properties and the pedogenic processes forming the soil as well as anthropogenic factors [45].

Magnetic susceptibility (χ) is a parameter proportional to the concentration of magnetic particles present in the soil. The value of the frequency susceptibility (χfd%) indicates the presence of superparamagnetic grains formed in the process of pedogenesis in the soil. The humus layer is characterized by the greatest dynamics of biochemical processes, which directly affect the production of strong magnetic metal oxides [12–14,48]. The analysis of the magnetic parameter χ in the tested soils indicated differences between treatments from 34.0 to 65.8 $\times$ 10^{-8}·m^3·kg^{-1}. The coexistence of Fe with Zn and Cr as well as a significant positive correlation between these elements, magnetic susceptibility (Fe: $r = 0.79$; Zn: $r = 0.65$; Cr: $r = 0.62$) and TOC ($r = 0.95$) may indicate that sewage sludge and fly ash are sources of ferromagnetics. Simultaneously, the analysis of correlation coefficients indicates significant positive relationships between the analyzed elements from the group of heavy metals. An inverse relationship (negative correlation) was shown between χ and Pb content. A similar tendency was found among the rest of the heavy metals and lead. The values of correlation coefficients among them were negative, higher than −0.7. There was also a negative correlation between the amount of lead and the TOC content ($r = -0.70$). This may indicate that the source of this element was primarily the fly ash. Scientific research also shows [13,48] that the magnetic susceptibility is strongly correlated with soil texture, pH and the content of organic matter, as well as the quantity and quality of HS in the agrosystem. Furthermore, Senesi and Loffredo [47] and Aijun et al. [49] indicate that HS play a special role in metal transformation. On the one hand, high molecular substances with the character of HAs increase their sorption in soil complexes and, on the other hand, FA contributes to their increased mobility. These processes are strongly correlated with the chemical properties of the soil environment and its biological activity [35,37,49,50]. The formation of specific forms of HS in agricultural soils depends on their essential physicochemical and chemical properties and the way in which they are used [36]. In the analyzed soils, we observed a different accumulation of TOC and a quantitative differentiation of HS strongly correlated with the scheme of the experiment. One of the soil indicators of HS' transformation is the humic acid/fulvic acid ratio (HA/FA), which indicates the diversity of HS and the direction of the humification process of the external organic matter. The intensity of the transformation process of organic matter in soils is expressed by an increased value of this parameter.

The organic matter transformation in the soil of experimental treatments led to the creation of various forms of HS depending on the variant of the study (Table 2), as evidenced by the HA/FA ratio. Among the analyzed soils, the predominance of HA was characterized by the organic matter of the treatments, which was enriched with fly ash. Thus, the direction of the organic carbon compound transformation leads to the formation of HS of diverse properties and different biological activity, as the biological research showed.

Table 2. HS in the studied treatments.

Number of Treatments	TOC [g·kg^{-1}]	SH	HA	HA/FA
		[%]		
1	10.69	28.4	14.06	0.98
2	11.17	28.6	13.71	0.92
3	11.01	28.2	13.74	0.95
4	14.73	34.15	17.96	1.11
5	13.82	35.05	18.23	1.16
6	13.95	35.03	17.94	1.05
LSD	2.26	4.40	2.91	0.12

LSD—low significant difference at $p = 0.05$; $n = 24$.

HS are the result of the biological activity of the soil. Their structure and properties determine the chelating properties and the protective role of humus in the natural environment. This can result in the retention of metals in the humic–mineral complexes, as indicated by high significant correlation coefficients (Table 3). Scientific research [38] strongly emphasizes that the number and survival of microorganisms in the soybean system depends on many factors: soil quality, in particular its fertility, pH, properties of root nodule bacteria and agrotechnics. Root nodule bacteria are natural components of soil microflora [23–25]. They occur typically everywhere but the number of their population is small. Therefore, in the case of growing legumes, a necessary agrotechnical treatment is seed inoculation with these bacteria [3–5]. *Bradyrhizobiaceae* inoculation which helps assimilate nitrogen is a simple and effective method of improving the efficiency of soybean cultivation. Within the species of *Bradyrhizobium*, *B. japonicum* is the best model of successful symbiosis affecting the intensity of nitrogen fixation. Scientifically, determining the number of *B. japonicum* can be considered as the most important method for determining the quality of a habitat for soybean cultivation. Soil is a complex environment, with numerous, complicated and often overlapping biological processes taking place. They mainly result from the presence of rich and diverse soil microbiota and interactions between them [21]. Sewage sludge and fly ash, due to the considerable amounts of macro- and microelements, can affect microbiological changes and the number of microorganisms. In the conducted experiment, there were strong relationships between the tested treatments (applied in the dose of sewage sludge and fly ash) and the TNB, including *Bradyrhizobium* (Table 4). The application of sewage sludge and fly ash in the soil in various quantitative combinations significantly influenced the value of the TNB in the soil in relation to the control (Figure 1). The number of these microorganisms in the control treatment from 2.74×10^7 cfu/g s.m. to 1.13×10^8 cfu/g s.m. in the treatment No. 3 (160 Mg·ha^1 of the sewage sludge application) was observed. Simultaneously, the number of *Bradyrhizobium* indicated various quantitative differences between variants of the experiment in comparison with the TNB (Figure 2). The biggest amount of bacteria of this type was found in the treatments (No 6) with the application of fly ash alone (7.18×10^3 cfu/g d.m.). A significantly lower content of *Bradyrhizobium* in treatments with sewage sludge at doses of 80 and 160 Mg·ha^1 (0.92×10^3 cfu/g d.m. and 1.09×10^3 cfu/g d.m., respectively) was observed, which shows a strong biotic interaction between the microflora of the sewage sludge and *Bradyrhizobium* (antagonistic relationships).

Table 3. Correlation between the growth of TNB, (cfu/g) *Bradyrhizobium* (cfu/nodule) and Fe (%) and other heavy metals' (mg·kg^{-1}) concentrations.

Feature	*Bradyrhizobium*	TNB
TOC	0.90	0.36
Fe	0.57	ns
Pb	−0.66	ns
Cr	ns	0.89
Cd	0.65	ns
Ni	0.77	0.38
Hg	−0.64	−0.43
pH	0.69	ns
χ	0.68	0.60
Λ	0.79	ns

n = 24; significant at—*p* = 0.001(above value 0.485); significant at—*p* = 0.05 (above value 0.343); ns—not significant.

Table 4. Relationships between the tested treatments (applied in the dose of sewage sludge and fly ash), the TNB and the separate *Bradyrhizobium*.

No. of Treatments	TNB (cfu/g)		*Bradyrhizobium* (cfu/Root Nodule)	
1	$2.74 \times 10^7 \pm 0.72$	B	$4.22 \times 10^3 \pm 0.45$	b
2	$4.75 \times 10^7 \pm 1.73$	B	$0.92 \times 10^3 \pm 0.05$	b
3	$1.13 \times 10^8 \pm 1.74$	A	$1.09 \times 10^3 \pm 0.36$	b
4	$6.82 \times 10^7 \pm 3.15$	C	$6.72 \times 10^3 \pm 0.35$	a
5	$1.09 \times 10^8 \pm 3.87$	A	$5.26 \times 10^3 \pm 2.67$	c
6	$3.14 \times 10^7 \pm 1.34$	B	$7.18 \times 10^3 \pm 2.69$	a

Data indexed by the same letter are statistically not significantly different (*p* = 0.05) in the same column.

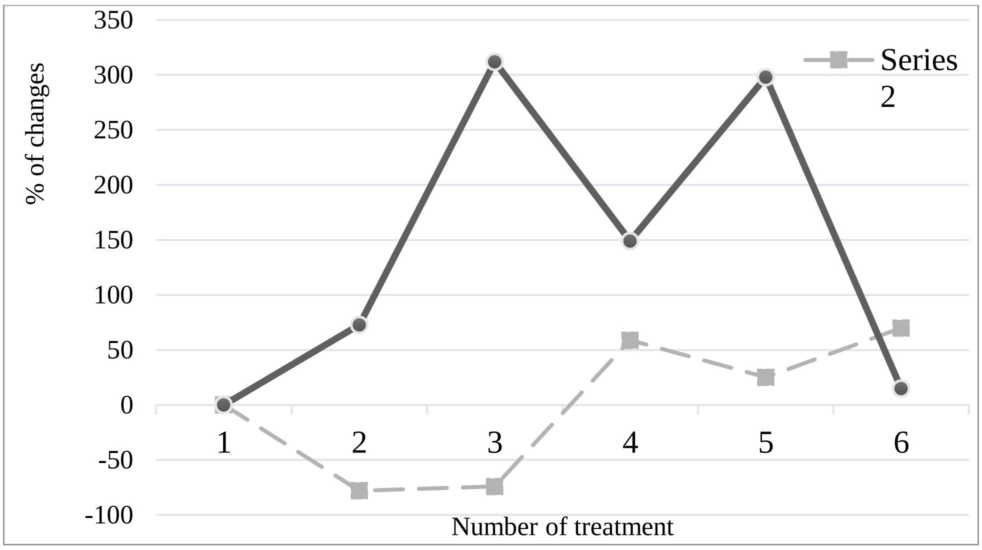
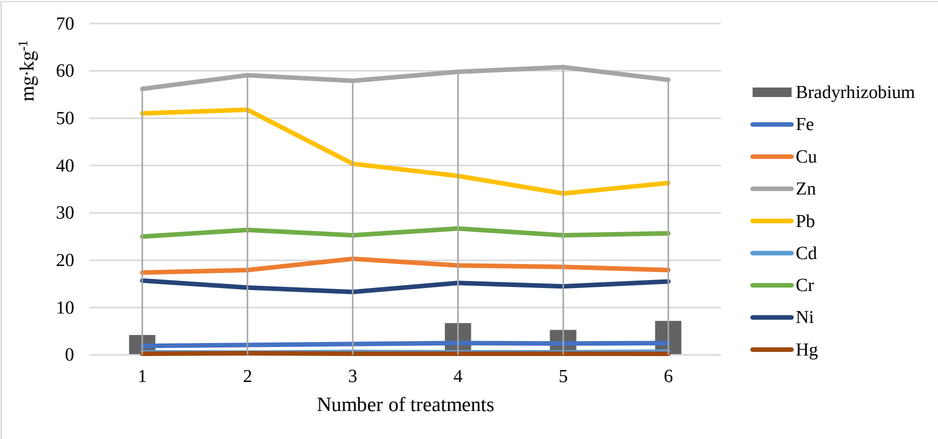
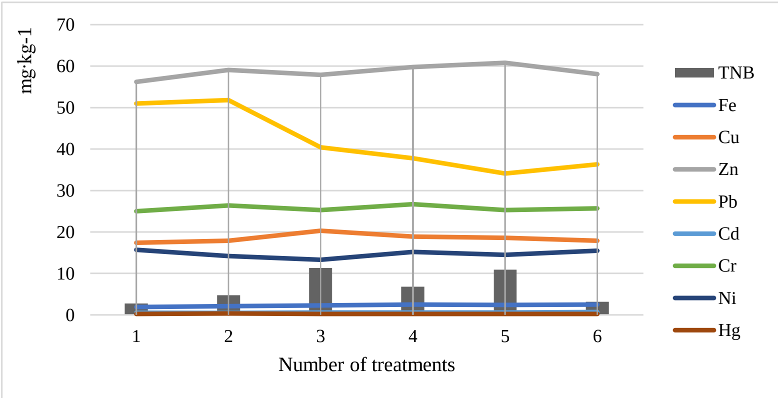


Figure 1. Effects of experiment variant on bacterial growth. Serie 1- TNB-($\times 10^7$ in cfu/g); Serie 2- *Bradyrhizobium* ($\times 10^3$ in cfu/root nodule).



(**a**) Correlation between Bradyrhizobium and heavy metals content



(**b**) Correlation between TNB and heavy metals content

Figure 2. Effects of heavy metals content on bacterial growth. TNB ($\times 10^7$ in cfu/g). *Bradyrhizobium* ($\times 10^3$ in cfu/root nodule).

Sewage sludge is a source of HS which can consist of different fractions with different weight and solubility. As Chen [51] showed, the lengthening of roots and stimulation of the development of secondary roots were observed for HS extracted from waste materials (including sewage sludge and fly ash) and added to nutrient solutions. Studies on the impact of organic matter, especially humic substances, on microorganisms and also plant growth, show positive effects on their biomasses. A small fraction of lower-molecular-weight components in HS can be taken up by soil organisms and effect the activity, quality and quantity of their community. Knowledge about the relationships between organic matter and its complexes with inorganic components and the rhizosphere community can help us to understand the relationships between them. Our study confirmed the influence of organo-mineral complexes on microbes' presence and showed differences between treatments. Compared with the control, the treatment with sewage sludge (160 Mg/ha) showed the biggest increase in TNB (by 312%) and the lowest (by 15%) in the treatment with fly ash alone (40 Mg/ha). The calculation of *Bradyrhizobium* numbers (in cfu/root nodule) showed differences in TNB (cfu/g of soil). The highest increase was observed in the treatment with fly ash (40 Mg/ha) and the treatment with sewage sludge and fly ash (80 Mg/ha + 40 Mg/ha), respectively, by 70% and 59%.

Soil pH is recognized as an important factor for diversity of the microorganisms [52–54]. Biological transformations of nitrogen compounds, especially nitrification processes, are very sensitive to changes in pH. Relationships between soil environmental properties, especially pH, showed, in general, the tolerance of different species communities to a change in pH. The ideal pH for the nitrification process is between 6.6–8.0, which can be strongly lowered to under 6.0. The optimum value of pH creates symbiosis between soybean and

Bradyrozobium spp. In acidic soils, symbiosis is limited by the excessive concentrations of aluminum and manganese ions. The higher value of pH (above 9.0) also stops the nitrification. The acidification of the soil also causes unfavorable changes in the quantitative and qualitative composition of the microflora community, increasing the fungal in relation to the bacterial, especially with actinomycetes, which decreases its fertility and the ability of bacteria to be involved in symbiotic and nitrogen assimilation processes. Considering the physicochemical and chemical properties of soils, it can be concluded that the number and activity of this group of microorganisms may be related to the pH of the soil and the availability of heavy metals. This dependence is indicated by the correlation coefficients between the analyzed parameters (Tables 4 and 5). Heavy metals cause toxic physiological and functional disorders in both microorganisms and plants. Pb is not biologically relevant but is toxic to cellular activities even at very low concentrations. However, many microorganisms have developed a stress tolerance mechanism that allows them to survive Pb exposure [20]. Cu and Ni are microelements for plants. Ni is an important component of urease in living cells [8,46]. Cu is an essential component of many biochemical reactions in living cells [45,51]. The study of Jaroslawiecka and Piotrowska-Seget [20] also shows that *B. japonicum* can be resistant to Pb by immobilizing phosphates.

Table 5. Correlation coefficient (*r*) between some soil parameters.

Feature											
	X						Y				
	Fe	**Cu**	**Zn**	**Pb**	**Cd**	**Cr**	**Ni**	**Hg**	**χ**	**pH**	**λ**
Fe											
Cu	0.43										
Zn	0.57	ns									
Pb	−0.79	−0.43	ns								
Cd	0.67	ns	ns	−0.78							
Cr	0.38	ns	0.51	Ns	ns						
Ni	ns	0.77	ns	Ns	ns	ns					
Hg	ns	0.52	0.64	0.78	−0.73	0.61	0.42				
χ	0.789	ns	0.65	−0.57	0.39	0.62	0.41	ns			
pH	0.84	ns	0.57	−0.80	0.74	ns	ns	−0.47	0.63		
λ	0.70	ns	0.65	−0.70	0.58	ns	ns	−0.46	0.57	0.96	
TOC	0.79	ns	0.66	−0.70	0.52	0.49	0.48	−0.40	0.95	0.79	0.78

$n = 24$; significant at—$p = 0.001$ (above value 0.485); significant at—$p = 0.05$ (above value 0.343); ns—not significant.

In our experiment, a significant effect of the analyzed heavy metals was the increase in the TNB (with the exception of chromium), including *Bradyrhizobium*. The number of *Bradyrhizobium* showed sensitivity to lead and mercury. A higher lead and mercury content decreased their number (Figure 2a). In the case of the total number of bacteria, a decrease in the bacteria numbers was found only in correlation with mercury (Figure 2b). Dependencies between other metals from the group of trace elements indicate a difference in the impact on bacterial metabolism (Tables 4 and 5).

The research of Tang et al. [40] shows that the combined application of organic matter with an inorganic fertilizer was a beneficial management practice for increasing soil fertility and bacterial resistance to xenobiotics in a cropping agrosystem.

4. Conclusions

The reduction in HS, frequently observed in Polish soils, constitutes a significant indicator of soil degradation. Supplementing the soil with organic matter is therefore necessary and many types of organic wastes, including sewage sludge, are used for this purpose. The fertilization effects of sewage sludge depend on many factors: chemical composition, dozes, mixtures with other ingredients, such as fly ash, etc. Its nutritional components can be utilized by cultivated plants and its organic matter can improve the balance of HS. The sewage sludge can serve as a universal fertilizer, if it is properly prepared, because it contains a large amount of organic matter as well as macro- and microelements, which are necessary for microorganisms. The dynamics of organic matter transformation processes are gradual, depending on chemical and biological environmental conditions. Soybean is a plant that lives with the species of nodule bacteria *Bradyrhizobium japonicum*, which is not found in Polish soils. Despite being treated with a bacterial vaccine, soybean plants very often require nitrogen enrichment in the soil. Nitrogen assimilation in legumes begins only in the phase of 3–4 leaves, so they require access to easily assimilable forms of nitrogen in the soil. Soil organic matter, including external organic matter (e.g., sewage sludge), is a store of nutrients for plants and microorganisms. Due to increasing prices of mineral fertilizers and a decrease in livestock production reducing the availability of manure, the agricultural use of organic waste materials seems to be the most reasonable.

Our study showed that:

1. The efficiency of soybean cultivation is related to the inclusion of chemical and biological methods in the agricultural technology cycle. In such a system of cultivation, the application of fertilizers and soil conditioners should be subordinated to the biological activity of the habitat, mainly due to the economics of production and the quality of the environment.
2. The transformation processes of organic matter in the soil of the experimental treatments caused the quantitative differentiation of the fractions of HS depending on the applied fertilization variant. The highest content of HA was detected in the soil treated with fly ash.
3. The application of sewage sludge and fly ash in the experimental treatments did not result in the amount of heavy metals exceeding the limit values for uncontaminated soils. In comparison to the control, a significant enrichment in iron, zinc and chrome of soil fertilized with sewage sludge, fly ash and fly ash treatments was detected. The lowest Hg content was observed in a treatment with fly ash only, with significant differences between those containing this element.
4. Negative values of correlation coefficients (higher than -0.7) between lead and other elements from the group of heavy metals and TOC content may indicate that the source of these elements was primarily the fly ash used in the experiment.
5. The coexistence of Fe with Zn and Cr and a significant positive correlation between them, magnetic susceptibility and TOC may indicate that sewage sludge and fly ash can be sources of ferromagnetic agents.
6. The results showed large physiological diversity and resistance of microorganisms, including *Bradyrhizobium*, to heavy metals in the applied fertilization variants.

Author Contributions: I.P. designed the experiments; I.P. performed the laboratory analysis of soil; K.G. performed the biological tests; I.P. and K.G. analyzed the data; I.P. and K.G. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the University of Opole.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data reported in this study are contained within the article.

Acknowledgments: We would like to thank Marta Bednarczyk, manager of Variety Assessment Experimental Station of Glubczyce, for providing soil and plant material for this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bednarczyk, M.; Pisarek, I. A sustainable development for soya production based on field research carried out in the variety assessment experimental station of Glubczyce in years 2015–2017. *Econ. Environ. Stud.* **2018**, *18*, 1219–1233. [CrossRef]
2. Dobek, T.K.; Dobek, M. Effectiveness of soybean production under the conditions of Poland (in Polish). *Agric. Eng.* **2008**, *4*, 233–240. Available online: https://bibliotekanauki.pl/articles/291262 (accessed on 14 April 2020).
3. Hama, J.R.; Kolpin, D.W.; LeFevre, G.H.; Hubbard, L.E.; Powers, M.M.; Strobel, B.W. Exposure and transport of alkaloids and phytoestrogens from soybeans to agricultural soils and streams in the Midwestern United States. *Environ. Sci. Technol.* **2021**, *55*, 11029–11039. [CrossRef] [PubMed]
4. Wenda-Piesik, A.; Ambroziak, K. The choice of soybean cultivar alters the underyielding of protein and oil under drought conditions in Central Poland. *Appl. Sci.* **2022**, *12*, 7830. [CrossRef]
5. Adesemoye, A.O.; Kloepper, J.W. Plant-microbes interactions in enhanced fertilizer-use efficiency. *Appl. Microbiol. Biotechnol.* **2009**, *85*, 1–12. [CrossRef] [PubMed]
6. Kaffine, D.T. Microclimate effects of wind farms on local crop yields. *J. Environ. Econ. Manag.* **2019**, *96*, 159–173. [CrossRef]
7. Pisarek, I.; Grata, K. The influence of the organic matter of sewage sediments on biological activity of microorganisms which carry out the transformations of carbon and nitrogen compounds. *Pol. J. Microbiol.* **2013**, *62*, 445–452. [CrossRef]
8. Pisarek, I. *The Influence of Sewage Sludge on the Direction of the Humic Substances Transformation and Some Soil Fertility of the Cambisol Developed from Silt (in Polish)*; University of Opole Press: Opole, Poland, 2007; p. 130.
9. Pisarek, I.; Glowacki, M.; Czernia, M. The impact of *Pleurotus ostreatus* on organic matter transformation processes. *Water Pollut. Res.* **2012**, *66*, 2666–2673. [CrossRef]
10. Nakano, K.; Matsumura, M. Utilization of dehydrated sewage sludge as an alternative nutrient to stimulate lipid waste degradation by the thermophilic toxic process. *J. Biosci. Bioeng.* **2002**, *94*, 113–118. [CrossRef]
11. Wlasniewski, S. Effect of fertilization with fly ash from black coal on some chemical properties of sandy soil and yields of oat. *Environ. Prot. Nat. Resour.* **2009**, *41*, 479–488. [CrossRef]
12. Evans, M.E.; Heller, F. Magnetism in the biosphere. *Int. Geophys.* **2003**, *86*, 185–210. [CrossRef]
13. Dytlow, S.K.; Gorka-Kostrubiec, B. Magnetic parameters as an indicator of the processes occurring in the degraded Chernozem developed in loess from Miechow area. *Sci. Rev. Eng. Env. Sci.* **2014**, *64*, 170–184. Available online: http://iks_pn.sggw.pl/eng_mp.html (accessed on 14 April 2020).
14. Zawadzki, J.; Magiera, T.; Fabijanczyk, P.; Kusza, G. Geostatical 3-dimensional integration of measurements of soil magnetic susceptibility. *Environ. Monit Assess* **2012**, *184*, 3267–3278. [CrossRef] [PubMed]
15. Fenta, B.A.; Driscoll, S.P.; Kunert, K.J.; Foyer, C.H. Characterization of drought-tolerance traits in nodulated soya beans: The importance of maintaining photosynthesis and shoot biomass under drought-induced limitations on nitrogen metabolism. *J. Agron. Crop Sci.* **2012**, *198*, 92–103. [CrossRef]
16. Żarski, J.; Kuśmierek-Tomaszewska, R.; Dudek, S.; Kropkowski, M.; Kledzik, R. Identifying climatic risk to soybean cultivation in the transitional type of moderate climate in Central Poland. *J. Cent. Eur. Agric.* **2019**, *20*, 143–156. [CrossRef]
17. Viteri, S.E.; Schmidt, E.L. Ecology of indigenous soil rhizobia: Selective response of *Bradyrhizobium japonicum* to a soybean meal. *Appl. Soil Ecol.* **1996**, *3*, 187–195. [CrossRef]
18. Berninger, T.; Lopez, O.G.; Bejarano, A.; Preininger, C.; Sessitsch, A. Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microb. Biotechnol.* **2018**, *11*, 12880. [CrossRef]
19. Jankowska, M.; Swedrzynska, D. Analysis of the interactions of microorganisms in soil environment (in Polish). *Kosmos* **2016**, *65*, 49–55. Available online: http://kosmos.icm.edu.pl/PDF/2016/49.pdf (accessed on 14 April 2020).
20. Jaroslawiecka, A.; Piotrowska-Seget, Z. Lead resistance in microorganisms. *Microbiology* **2014**, *160*, 12–25. [CrossRef]
21. Smith, C.R.; Blair, P.L.; Boyd, C.; Cody, B.; Hazel, A.; Hedrick, A.; Kathuria, H.; Khurana, P.; Kramer, B.; Muterspaw, K.; et al. Microbial community responses to soil tillage and crop rotation in a corn/soybean agroecosystem. *Ecol. Evol.* **2016**, *6*, 8075–8084. [CrossRef]
22. Young, I.M.; Ritz, K. Tillage, habitat space and function of soil microbes. *Soil Tillage Res.* **2000**, *53*, 201–213. [CrossRef]
23. Agri, U.; Chaudhary, P.; Sharma, A.; Kukreti, B. Physiological response of maize plants and its rhizospheric microbiome under the influence of potential bioinoculants and nanochitosan. *Plant Soil* **2022**, *474*, 451–468. [CrossRef]
24. Chaudhary, P.; Sharma, A.; Chaudhary, A.; Khati, P.; Gangola, S.; Maithani, D. Illumina based high throughput analysis of microbial diversity of maize rhizosphere treated with nanocompounds and *Bacillus* sp. *Appl. Soil Ecol.* **2021**, *159*, 103836. [CrossRef]
25. Chaudhary, P.; Chaudhary, A.; Bhatt, P.; Kumar, G.; Khatoon, H.; Rani, A.; Kumar, S.; Sharma, A. Assessment of Soil Health Indicators Under the Influence of Nanocompounds and *Bacillus* spp. in Field Condition. *Front. Environ. Sci.* **2022**, *9*, 769871. [CrossRef]
26. Tu, T.-C.; Lin, S.-H.; Shen, F.-T. Enhancing symbiotic nitrogen fixation and soybean growth through co-inoculation with *Bradyrhizobium* and *Pseudomonas* isolates. *Sustainability* **2021**, *13*, 11539. [CrossRef]

27. Cadore, L.S.; Vey, R.T.; Fresinghelli, J.C.F.; Dotto, L.; Mendes, F.B.; Silva, A.C.F. *Trichoderma* and *Bradyrhizobium japonicum* bioformulates bioformulates on soy initial growth. *Cienc. Nat. Santa Maria* **2020**, *42*, e22. [CrossRef]
28. Röskeą, V.M.; Guimarães, V.F.; Brito, T.S.; Lerner, A.W.; Junior, R.C.; Silva, A.S.L.; Ankla, M.A. Inoculation and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* in soybean crop with the use of soil bioactivator. *Commun. Plant Sci.* **2022**, *12*, 24–32.
29. Jabborova, D.P.; Enakiev, Y.I.; Davranov, K.D.; Begmatov, S.A. Effect of co-inoculation with *Bradyrhizobium japonicum* and *Pseudomonas putida* on root morph-architecture traits, nodulation and growth of soybean in response to phosphorus supply under hydroponic conditions. *Bulg. J. Agric. Sci.* **2018**, *24*, 1004–1011.
30. Bulegon, G.L.; Guimarães, V.F.; Klein, J.; Battistus, A.G.; Inagaki, A.M.; Offemann, L.C.; Souza, A.K.P. Enzymatic activity, gas exchange and production of soybean co-inoculated with *Bradyrhizobium* japonicum and Azospirillum brasilense. *AJCS* **2017**, *11*, 888–896. [CrossRef]
31. Carro, L.; Menendez, E. Knock, knock-let the bacteria in enzymatic potential of plant associated bacteria. In *Molecular Aspects of Plant Beneficial Microbes in Agriculture*, 1st ed.; Sharma, V., Salwan, R., Tawfeeq, L., Eds.; Academic Press; Elsevier: London, UK, 2020; Chapter 13; pp. 169–178.
32. Arana, I.; Orruńo, M.; Barcina, I. How to solve practical aspects of microbiology. In *Part 2: Basic Methods for Microbial Enumeration*; University of Basque Country: Biscay, Spain, 2013.
33. Grata, K.; Rombel-Bryzek, A.; Ziembik, Z. *Bacillus subtilis* BS-2 and Peppermint Oil as Biocontrol Agents Against *Botrytis cinerea*. *Ecol. Chem. Eng. S* **2019**, *26*, 597–607. [CrossRef]
34. Saeed, Q.; Xiukang, W.; Haider, F.U.; Kučerik, J.; Mumtaz, M.Z.; Holatko, J.; Naseem, M.; Kintl, A.; Ejaz, M.; Naveed, M. Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: A comprehensive review of effects and mechanisms. *Int. J. Mol. Sci.* **2021**, *22*, 10529. [CrossRef] [PubMed]
35. Moliszewska, E.B.; Pisarek, I. Influence of humic substances on the growth of two phytopathogenic soil fungi. *Environ. Int.* **1996**, *22*, 579–582. [CrossRef]
36. Pisarek, I. Characterization of humic substances formed in soil fertilized with sewage sludge and cattle manure. *Humic Subst. Ecosyst.* **2003**, *5*, 93–99. Available online: https://scholar.google.pl (accessed on 14 April 2020).
37. Etesami, H. Bacterial mediated alleviation of heavy metal stress and decreased accumulation of metals in plant tissues: Mechanisms and future prospects. *Ecotoxicol. Environ. Saf.* **2018**, *147*, 175–191. [CrossRef] [PubMed]
38. Seneviratne, S.; Gunaratne, T.; Bandara, L.; Weerasundara, L.; Rajakaruna, N.; Seneviratne, G.; Vithanage, M. Plant growth promotion by *Bradyrhizobium japonicum* under heavy metal stress. *South Afr. J. Bot.* **2016**, *105*, 19–24. [CrossRef]
39. Wani, P.A.; Khan, M.S.; Zaidi, A. Effect of metal tolerant plant growth promoting *Bradyrhizobium* sp. (*vigna*) on growth, symbiosis, seed yield and metal uptake by greengram plants. *Chemosphere* **2007**, *70*, 36–45. [CrossRef] [PubMed]
40. Tang, H.; Xu, Y.; Li, C.; Shi, L.; Wen, L.; Li, W.; Cheng, K.; Xiao, X. Effects of different long-term fertilizer management systems on soi microbial biomass turnover in a double-cropping rice field in Southern Cina. *Agriculture* **2022**, *12*, 1662. [CrossRef]
41. Humate solubility determination. In *American Colloid Company Procedure 3009*; American Colloid Company: Hoffman Estates, IL, USA, 1998.
42. Goluchowska, B.; Strzyszcz, Z.; Kusza, G. Magnetic susceptibility and heavy metal content in dust from the lime plant and cement plant in Opole voivodship. *Arch. Environ. Prot.* **2012**, *38*, 71–80. [CrossRef]
43. Graham, P.H. Selective medium for growth of Rhizobium. *Appl. Microbiol.* **1969**, *17*, 769–770. [CrossRef]
44. Penna, C.; Massa, R.; Olivieri, F.; Gutkind, G.; Cassán, F. A simple method to evaluate the number of Bradyrhizobia on soybean seeds and its implication on inoculant quality control. *AMB Express* **2011**, *19*, 1–21. [CrossRef]
45. Kabata-Pendias, A. *Trace Elements in Soils and Plants*; CRC Press: Boca Raton, FL, USA, 2010; p. 507. Available online: https://www.crcpress.com (accessed on 14 April 2020).
46. Polacco, J.C.; Mazzafera, P.; Tezotto, T. Opinion—Nickel and urease in plants: Still many knowledge gaps. *Plant Sci.* **2013**, *199–200*, 79–90. [CrossRef] [PubMed]
47. Senesi, N.; Loffredo, N. Interaction with metals. In *Encyclopedia of Soils in the Environment*; Elsevier: Amsterdam, The Netherlands, 2005; pp. 101–112. [CrossRef]
48. Jordanova, D.; Jordanova, N.; Petrov, P.; Tsacheva, T. Soil development of three Chernozem-like profi les from North Bulgaria revealed by magnetic studies. *Catena* **2010**, *83*, 158–169. [CrossRef]
49. Aijun, Y.; Changle, Q.; Shusen, M.; Reardon, E.J. Effects of humus on the environmental activity of mineral-bound Hg: Influence on Hg volatility. *Appl. Geochem.* **2006**, *21*, 446–454. [CrossRef]
50. Rombel-Bryzek, A.; Pisarek, I. The effect of humic acids on metabolic activity of sugar beet under drought conditions (in Polish). *Proc. ECOpole* **2017**, *11*, 279–286. [CrossRef]
51. Chen, Y.; Aviad, T. Effects of Humic Substances on plant growth. In *Humic Substances in Soil and Crop Sciences: Selected Readings*; MacCarthy, P., Clapp, C.E., Malcolm, R.L., Bloom, P.R., Eds.; American Society of Agronomy, Inc.: Madison, WI, USA, 1990; Chapter 7; pp. 161–186. [CrossRef]

52. Florent, P.; Chauchie, H.M.; Herold, M.; Jacquet, S.; Ogorzaly, L. Soil pH, calcium content and bacteria as major factors responsible for the distribution of the known fraction of the DNA bacteriophage populations in soils of Luxemburg. *Microorganisms* **2022**, *10*, 1458. [CrossRef] [PubMed]
53. Rousk, J.; Bååth, E.; Brookes, P.; Lauber, C.; Lozupone, C.; Caporaso, J.G.; Knight, R.; Fierer, N. Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J.* **2010**, *4*, 1340–1351. [CrossRef]
54. Wang, C.; Zhou, X.; Guo, D.; Zhao, J.; Yan, L.; Feng, G.; Gao, Q.Y.H.; Zhao, L. Soil pH is the primary factor driving the distribution and function of microorganisms in farmland soils in northeastern. *Ann. Microbiol.* **2019**, *69*, 1461–1473. [CrossRef]